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CCR5 antagonism by maraviroc inhibits Hodgkin lymphoma microenvironment interactions and xenograft growth

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Abstract

Classic Hodgkin lymphoma tumor cells express a functional CCR5 receptor, and tumor tissues express high CCL5 levels, suggesting that CCL5-CCR5 signaling is involved in tumor-microenvironment formation and tumor growth.

Using the CCR5 antagonist maraviroc and a neutralizing anti-CCL5 antibody, we found that CCL5 secreted by Classic Hodgkin lymphoma cells recruited Mesenchymal-stromal cells and monocytes. “Education” of Mesenchymal-stromal cells by tumor cell conditioned medium enhanced Mesenchymal-stromal cells proliferation and CCL5 secretion. In turn, educated Mesenchymal-stromal cell conditioned medium increased the clonogenic growth of tumor cells and monocyte migration, but these effects were reduced by maraviroc. Monocyte education by tumor cell conditioned medium induced their growth and reprogrammed them towards immunosuppressive tumor-associated-macrophages that expressed IDO and PD-L1 and secreted IL-10, CCL17 and TGF- β . Educated-monocyte conditioned medium slowed the growth of phytohemagglutinin-activated lymphocytes. Maraviroc decreased tumor cell growth and synergized with doxorubicin and brentuximab vedotin. A three-dimensional heterospheroid assay showed that maraviroc counteracted both the formation and viability of heterospheroids generated by co-cultivation of tumor cells with Mesenchymal-stromal cell and monocytes. In mice bearing tumor cell xenografts, maraviroc reduced tumor growth by more than 50% and inhibited monocyte accumulation, without weight loss. Finally, in classic Hodgkin lymphoma human tumor tissues, CCL5 and CD68 expression correlated positively, and patients with high CCL5 levels had poor prognosis.

In conclusion, since the present challenges are to find molecules counteracting the formation of the immunosuppressive tumor microenvironment or new, less toxic drug combinations, the repurposed drug maraviroc may represent a new opportunity for classic Hodgkin lymphoma treatment.

Introduction

Inflammatory chemokines are indispensable “gate-keepers” of inflammation and immunity against cancer, but tumor cells can subvert chemokines into acting as tumor-promoting molecules.¹ C-C motif chemokine ligand 5 (CCL5) is one such chemokine that can favor tumor development in multiple ways, for example by acting as a growth factor for tumor cells, stimulating angiogenesis, recruiting stromal and inflammatory cells, and taking part in immune evasion mechanisms.²⁻⁶ CCL5 belongs to the C-C chemokine family whose members include CCL3 and CCL4.^{1,2} Its activity is mediated through binding to CCR1, CCR3, and CCR5, while CD44 serves as an auxiliary receptor.²

CCL5 and other chemokines are expressed at higher levels in classic Hodgkin lymphoma (cHL) tumor tissues than in healthy lymph nodes and in tissues with reactive lymphoid hyperplasia.^{7 8} Both CCL5 and its receptor CCR5 are constitutively expressed by cHL-derived cell lines,⁷ by tumor cells from cHL lymph node tissues, and by bystander cells including stromal cells and lymphocytes.⁷ The CCR5 receptor expressed by cHL cells is fully functional and its ligands function as both paracrine and autocrine growth factors.⁷

The interactions of cHL tumor cells with a variety of non-tumor reactive cells accumulating in cHL tissues mediate tumor cell growth, formation of an immunosuppressive, protective tumor

microenvironment (TME), neo-angiogenesis⁹ and drug resistance.^{10,11} Increasing evidence suggests that not only T cells,¹² but also mesenchymal stromal cells (MSCs)¹³ and monocytes^{14,15} contribute to the TME in cHL.^{11,16} MSCs, by modulating NKG2D expression in T cells and its ligand in tumor cells, reduce the immune response against cHL cells.¹³ A high number of infiltrating macrophages,^{17,18} predominantly derived from circulating monocytes,¹⁹ and a high absolute monocyte count in peripheral blood both correlate with poor cHL prognosis.^{20,21} These observations likely reflect the ability of cHL cells to reprogram macrophages towards immunosuppressive tumor-associated macrophages (TAMs).^{20,21}

Given current knowledge about cell-cell interactions in cHL, there is interest in drugs that can interfere with this crosstalk.²²⁻²⁵ But since drug discovery is expensive and time-consuming, drug repurposing is an attractive approach for finding new cancer treatments.²⁶ One such repurposed drug is the CCR5 antagonist maraviroc.²⁷ Approved by the US Food and Drug Administration for the treatment of HIV infection, maraviroc causes few side effects in humans, even during long-term therapy.^{28,29} As an anticancer drug, maraviroc has different effects: it blocks metastasis of basal breast cancer cells;³⁰ it decreases the migration of regulatory T cells; it reduces metastatic breast cancer growth in the lungs;³¹ and it inhibits the accumulation of fibroblasts in human colorectal cancer.³² Maraviroc reprograms immunosuppressive myeloid cells and reinvigorates antitumor immunity by targeting the autocrine CCL5-CCR5 axis in bone marrow.⁶ It also polarizes macrophages towards an M1-like functional state.²⁷

Our working hypothesis is that cHL cancer cells, by secreting CCL5, recruit both MSCs and monocytes to the TME, and then reprogram these cell types to make them pro-tumorigenic. Thus,

blocking the CCR5 receptor should inhibit not only tumor growth, as we previously observed,⁷ but also the recruitment of cells to form the protective, immunosuppressive TME.

Here, we investigated the role of CCL5-CCR5 signaling in the interactions of monocytes and MSCs with cHL cells, using in particular three-dimensional multicellular heterospheroids³³ formed by tumor cells, monocytes and MSCs, as well as an *in vivo* cHL model and tissues from cHL patients.

Methods

Maraviroc (Sigma-Aldrich) was dissolved in DMSO at 51.8 mM. Other reagents are detailed in Supplementary methods, together with protocols for cell migration, proliferation, clonogenic growth and senescence assays, immunosuppression, synergy, flow cytometry, ELISAs and other cell-based assays, immunofluorescence, survival of tumor xenografts and statistical analysis.

Cell culture and conditioned media

Authenticated cHL-derived cell lines L-1236, L-428, KM-H2, HDLM-2, and L-540 (DSMZ, Germany) were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS). To prepare conditioned medium (CM) from cHL cell lines, cells were seeded at 2.0×10^5 /ml in RPMI-1640 plus 10% FCS, and medium collected after 72 h. Human bone marrow-derived and adipose tissue-derived mesenchymal stromal cells (MSCs) (BM-MSCs and AT-MSC, respectively) were purchased from Lonza (Verviers, Belgium). cHL-MSCs (cHL-MSCs) from frozen lymph nodes were generated as described in Supplementary methods. BM-MSCs, AT-MSC and cHL-MSCs were maintained in Mesenchymal-Stem-Cell Growth Medium Bulletkit (Lonza) to avoid differentiation.

Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) from healthy donor blood using CD14 Microbeads, Human (Miltenyi Biotec). To generate tumor-educated MSCs (E-MSCs) and monocytes (E-monocytes), MSCs and monocytes were cultured separately for 6 days in complete culture medium (RPMI-1640, 10% FCS) containing 20% CM from cHL cell lines; half the volume of medium was replaced every other day. To prepare CM from these tumor-educated cells, they were washed and cultured in fresh medium for 72 h.

3D culture of heterospheroids

Heterospheroids were generated by co-culturing various combinations of cHL cells , HL-MSCs and monocytes (1.0×10^4 /mL of each cell type) in RPMI-1640 medium containing 1% FCS, using plates coated with 20 mg/mL poly-HEMA (Sigma) to prevent adhesion. After 4 days, heterospheroids were tested for CCL5 secretion into the medium by ELISA. In some experiments, heterospheroids were treated with maraviroc, alone or with doxorubicin, for 6 days. Growth was evaluated using the PrestoBlue Cell Viability Reagent (Invitrogen).

Tumor xenograft experiments

Animal experiments were approved by the Italian Ministry of Health (no. 671/2015/PR). We used ten 4-week-old female athymic nude/nude mice (Harlan Laboratories) and ten 4-week-old male NOD/SCID gamma chain deficient (NSG) mice (Charles River). L-540 (20×10^6 cells/animal) and L-428 cells (10×10^6 cells/animal) were suspended in Matrigel (diluted 1:3 in PBS) and inoculated into the flank of nude mice (L540) or NSG mice (L-428). When tumors were palpable, animals were divided into two equal groups and treated every day (L-540) or every other day (L-428) with maraviroc (intraperitoneal injection, 10 mg/kg³⁴) or vehicle (PBS). Body weight and tumor volume were measured daily.

Immunohistochemistry tissue microarray analysis of cHL patients

The study protocol was approved by the institutional review board of University Medical Center Groningen. We recruited 65 patients with cHL (*Online Supplementary Table S1*). All study subjects provided written informed consent. Immunohistochemistry was performed for CCL5 (C-19 antibody, 1:200 dilution, Santa Cruz Biotechnology)(antigen retrieval in 10 mM Tris-HCl pH 9, 1 mM

EDTA). CD68 was detected with KP1 antibody (1:4000 dilution, Dako)(antigen retrieval in 10 mM citrate buffer, pH 6).

Results

Maraviroc inhibits crosstalk between cHL cells and both MSCs and monocytes

Conditioned medium from L-1236 and KM-H2 cHL-derived cell lines stimulated the migration of BM-MSCs (Figure 1A, left) and AT-MSCs (Figure 1A, right). Since MSCs express CCR5 (*Online Supplementary Figure S1A*)³⁵ and cHL cells secrete CCL5,⁷ we investigated if this chemokine is directly involved in MSC migration. Addition of the CCR5 antagonist maraviroc (Figure 1A) or a neutralizing anti-CCL5 antibody (Figure 1B) significantly reduced the MSC migration induced by cHL conditioned medium. Growth of MSCs from different sources (bone marrow, adipose tissue, and cHL lymph nodes) increased in the presence of cHL conditioned medium in a dose-dependent manner (Figure 1C and *Online Supplementary Figure S1B*). This effect was partially mediated by FGF2, TGF β 1 and TNF α secreted by cHL cells, since addition of antibodies against these growth factors significantly, but incompletely, reduced growth (Figure 1D). cHL conditioned medium almost totally abolished MSC senescence induced by serum starvation (*Online Supplementary Figure S1C*) and reduced apoptosis induced by doxorubicin treatment (*Online Supplementary Figure S1D*).

To survive and proliferate, cancer cells not only recruit but also shape or “educate” normal cells.¹¹ BM-MSCs released very low amounts of CCL5 under normal culture conditions (Figure 1E). After being cultured with cHL conditioned medium, thereby becoming tumor educated (E-BM-MSCs),

they strongly secreted CCL5 (Figure 1E). This response was partially reduced by treatment with an anti-TNF α antibody (Figure 1F). Education of BM-MSCs did not, however, induce the secretion of CCL3 or CCL4 (data not shown).

Conditioned medium from E-BM-MSCs increased the clonogenic growth of L-1236, HDLM-2 and L-540 cells, and this effect was reduced by maraviroc in a dose-dependent manner (Figure 1G and *Online Supplementary Figure S2A*). This effect was due to CCL5-CCR5 interactions, because it was partially inhibited by inclusion of a neutralizing anti-CCL5 antibody (*Online Supplementary Figure S2B*). Conditioned medium from BM-MSCs educated with L-1236 conditioned medium (and thereby containing CCL5; Figure 1E) increased the migration of CD14⁺ monocytes, and this effect was reduced in a dose-dependent manner by maraviroc (Figure 1H).

Taken together, these results suggest that E-BM-MSCs, by secreting CCL5, stimulate tumor growth as well as monocyte recruitment in the TME.

cHL cells induce monocyte migration and proliferation

cHL conditioned medium increased CCR5 expression in monocytes (*Online Supplementary Figure S3A*) and enhanced their migration through fibronectin-coated Boyden chambers (*Online Supplementary Figure S3B*). Representative photomicrographs of transmigrated monocytes (red Fast-Dil colored cells) are shown in *Online Supplementary Figure S3C*. This enhanced monocyte migration was significantly reduced when maraviroc (Figure 2A) or a neutralizing anti-CCL5 antibody (Figure 2B) was added. cHL cells, especially L-1236 and L-428 cells, secreted macrophage colony-stimulating factor (M-CSF) (Figure 2C), a cytokine involved in monocyte proliferation and differentiation. In accordance, conditioned medium from cHL cells increased monocyte growth (Figure 2D and *Online*

Supplementary Figure S3D). E-monocytes secreted CCL3 and low amounts of CCL4 and CCL5 (*Online Supplementary Figure S3E*). Treatment of L-1236, HDLM-2 and L-540 cells with conditioned medium from E-monocytes increased the number of viable tumor cells (*Online Supplementary Figure S3F*) and stimulated clonogenic growth (Figure 2E), but this growth was inhibited by maraviroc treatment (Figure 2E and *Online Supplementary Figure S3G*).

Reprogramming of monocytes by cHL cells

TAMs express high levels of CD206, PD-L1 and IDO; they secrete IL-10, CCL17/TARC and TGF- β , and can inhibit growth of activated T cells.¹⁶ When monocytes were cultivated in conditioned medium from L-1236 or L-428 cells, they upregulated the secretion of IL-10, CCL17, and TGF β (Figure 3A) and increased the expression of CD206, PD-L1, and IDO (Figure 3B). Conditioned medium from L-540 cells did not induce IL-10 or CCL17 secretion or IDO expression by monocytes, but did enhance CD206, PD-L1, and especially TGF β secretion (Figures 3A-B). Conditioned medium from E-monocytes slowed, in a dose-dependent manner, the growth of phytohemagglutinin-activated lymphocytes (Figure 3C). These results demonstrate that cHL cells recruit, induce the proliferation and then reprogram monocytes towards an immunosuppressive phenotype.

Maraviroc slows tumor cell growth and synergizes with doxorubicin and brentuximab vedotin

Considering that cHL cells express a functional CCR5 receptor, we evaluated cHL cell growth in the presence of increasing concentrations of maraviroc. This treatment slightly slowed the growth of cHL cells (Figure 4A) and slightly increased the percentage of cells in the G1 phase of the cell cycle (Figure 4B and *Online Supplementary Figure S4A*). On the contrary, maraviroc treatment did not

induce apoptosis-necrosis as shown by the lack of change in annexin-V and 7-AAD staining (*Online Supplementary Figure S4B*) and in levels of activated caspase-3/7 (*Online Supplementary Figure S4C*). Maraviroc (25, 50, 100 μ M) synergized with doxorubicin and brentuximab vedotin as indicated by the combination index being <0.9 in all three conditions tested (Table 1 and *Online Supplementary Figure S5A-B*). Moreover, maraviroc exerted additive or antagonist effects (combination index ≥ 0.9) in combination with cisplatin, gemcitabine and vinorelbine (Table 1). Synergistic effects with both doxorubicin and brentuximab vedotin were also obtained using ten-fold lower maraviroc concentrations (2.5, 5, 10 μ M), but not with hundred-fold lower concentrations (0.25, 0.5, 1.0 μ M) (*Online Supplementary Table S2*).

Maraviroc inhibits 3D heterospheroid formation

To mimic TME interactions, we co-cultured cHL cells with MSCs and monocytes in a non-adherent, 3D setting in poly-HEMA-coated wells. The cells were dispersed in the medium at time 0 but at 24 h had started to self-assemble into 3D heterospheroids (Figure 4C and *Online Supplementary Figure S6A*). Heterospheroids containing all three cell types secreted high levels of CCL5, and those containing only cHL cells (either L-1236 or HDLM-2) and HL-MSCs also had high CCL5 secretion, whereas the combinations of just monocytes with cHL cells or with HL-MSCs expressed low levels (Figure 4D). However, when combined with HL-MSCs and cHL cells, monocytes were able to induce a further increase of CCL5 secretion (Figure 4D).

Next, we evaluated the effects of maraviroc on the self-assembling ability and viability of heterospheroids. Maraviroc inhibited the self-assembling of cHL, HL-MSCs and monocytes into heterospheroids (Figure 4E). It also reduced the total number of viable cells in the heterospheroids

(Figure 4F). Considering this reduced viability, and the finding that maraviroc synergized with doxorubicin (Table 1), we applied drug combinations to the heterospheroids and found that doxorubicin and maraviroc exerted synergistic activity (combination index <1) against heterospheroids too (*Online Supplementary Figure S6B*). When cHL cells (CD30+) were recovered from heterospheroids by trypsinization and purification with anti-CD30 beads, fewer viable tumor cells were recovered from maraviroc-treated than from untreated heterospheroids (*Online Supplementary Figure S6C*). The cells from treated heterospheroids produced fewer colonies (*Online Supplementary Figure S6D*) and were proportionately more in G1 than in G2M phase than untreated cells (*Online Supplementary Figure S6E*).

Maraviroc slows the growth of cHL tumor xenografts and reduces infiltrated TAMs

To analyze the anticancer activity of maraviroc *in vivo*, we studied L-540 tumor cell xenografts in female athymic nude mice, treated every day with an intraperitoneal injection of 10 mg/kg maraviroc or vehicle. By day 12, untreated tumors had grown to a mean volume of 880 mm³ (SD = 88 mm³), whereas maraviroc-treated tumors were more than 50% smaller (435±75 mm³; $P < 0.0001$, Student's *t* test; Figure 5A and *Online Supplementary Figure S7A*). Maraviroc treatment was not toxic, as the animals were normal to physical inspection and had similar weight to untreated animals (Figure 5B). Maraviroc-treated mice had significantly better “survival” (i.e. tumor volume <800 mm³) than untreated mice ($P = 0.002$, log rank test) (*Online Supplementary Figure S7B*). Since maraviroc inhibited the migration of monocytes *in vitro*, we evaluated if similar activity was also detectable *in vivo* by examining infiltrating TAMs (CD68+) in L-540 tumor xenografts. Immunofluorescence analysis of CD30 on tissue sections showed no difference between untreated and maraviroc-treated mice (*Online*

Supplementary Figure S7C). However, the animals differed substantially in staining for CD68, a marker of TAM infiltration, which was almost completely absent in maraviroc-treated mice (Figure 5C-D and *Online Supplementary Figure S7D*).

Similar results were obtained when male NSG mice were injected with L-428 tumor cells (Figure 5E-H). In particular, maraviroc treatment reduced xenograft growth by about 60% (Figure 5E), without weight loss (Figure 5F), and it reduced CD68 staining by 75% (Figure 5G-H and *Online Supplementary Figure S7D*).

High CCL5 expression positively correlates with CD68 and poor survival

To confirm our *in vitro* and *in vivo* results, we studied cHL tissues from 65 patients (*Online Supplementary Table S1*). cHL tissues had a median CCL5 expression level of 12% positive pixels (range, 0%-44%) and a median CD68 expression level of 17% positive pixels (range, 0%-58%) (Figure 6A). No difference in CCL5 expression was found in EBV-positive versus EBV-negative HL samples (data not shown). On the contrary, a significant correlation between CCL5 and CD68 levels was found (Spearman $r=0.251$, $P=0.0487$) (Figure 6B). Patients were then divided into three arbitrary categories of CCL5 expression: low (0%-10%), medium (11%-20%) and high (21%-100%). These groups had 24, 22, and 16 patients, respectively (data missing for 3 patients). A Kaplan-Meier plot showed that the group of patients with high CCL5 expression had significantly worse survival ($P=0.0072$) than patients with low or medium expression (Figure 6C). The hazard ratio for progression-free survival of low/medium CCL5 expression to that of high expression was 0.015 ($P=0.0016$; 95% CI, 0.0011-0.2064).

Discussion

The TME plays an active role in cHL,¹¹ suggesting the possibility of developing alternative treatment strategies that target not only tumor cells, but also the TME's protective effects.^{11,22} Here, we found that maraviroc, a CCR5 antagonist, inhibited cHL cell recruitment of monocytes and MSCs, reduced the cHL cell growth-promoting effects of CCR5 ligands secreted by monocytes and MSCs, synergized with doxorubicin and brentuximab vedotin, and decreased cHL tumor xenograft growth and monocyte infiltration *in vivo*. In cHL patients, high CCL5 levels correlated with monocyte infiltration and poor prognosis.

Our *in vitro* results suggest that there is a “domino effect” within the cHL TME: tumor cells, by secreting CCL5, among other molecules, recruit, expand, and educate MSCs and monocytes; these cells, in turn, secrete CCR5 ligands (i.e. CCL3 and CCL4) to recruit other normal cells and stimulate the growth of tumor cells, which reprogram (educate) monocytes to become immunosuppressive TAMs. A schematic view of the possible mechanisms leading to cHL cell proliferation and TME formation by CCR5 ligands, and the counteracting effects of maraviroc, is shown in Figure 7.

We found that conditioned medium from cHL cells increased the growth of MSCs from different sources, and maraviroc decreased MSC recruitment by cHL cells. Thus, the development of fibrosis in cHL tissues could be explained by recruitment of MSCs by cytokines, including CCL5, secreted by primary tumors and expansion or activation by FGF2, TGF β or TNF α secreted by tumor cells. Moreover, our findings that cHL education of MSCs (E-MSCs) increased the secretion of CCL5 and that maraviroc reduced monocyte recruitment by E-MSC conditioned medium suggest an active role of E-MSCs in TME formation. In this perspective, MSCs of the cHL TME not only may down-

regulate anti-tumor immune responses through NKG2D-NKG2DL interactions,^{36,37} but may also enhance the number of infiltrated TAMs by secreting CCL5 and, consequently, supporting tumor progression.

Macrophages seem to be involved in the pathogenesis of cHL, since high levels of TAMs, as well as the absolute monocyte count in the blood, correlate with an unfavorable clinical outcome.^{17,20,21,38} Despite the importance of monocyte levels in TME, their education (i.e. conditioning by tumor cells) seems an essential prerequisite for their pro-tumor activity.^{39,40} Recently, it was demonstrated that conditioned medium from cHL cell lines induced an immunosuppressive phenotype in macrophages obtained by pre-cultivation of monocytes with M-CSF or GM-CSF.¹⁶ Consistent with the finding that cHL cells secrete M-CSF, the education of monocytes by cHL cell conditioned medium, also without a preconditioning with M-CSF or GM-CSF,^{16,17} was sufficient to shape monocytes to secrete and express immunosuppressive molecules, including IDO and PD-L1, and to inhibit PHA-activated lymphocyte growth.

Recently, it was found that, in cHL tissues, TAMs are not randomly distributed; in fact, PD-L1+ TAMs lie in greater proximity to PD-L1+ tumor cells and PD1+ T cells preferentially localize in proximity to PD-L1+ TAMs, suggesting a model in which the inflammatory microenvironment of cHL is highly organized with PD-L1+ TAMs immediately surrounding Hodgkin and Reed-Sternberg cells to engage PD-1+ T cells and augment immune suppression.⁴¹ Thus, our results suggest that cHL cells, by inducing PD-L1 expression in monocytes, contribute to the building of the immunosuppressive niche.⁴¹ Maraviroc, by reducing monocyte recruitment, may counteract this phenomenon.

Since current two-dimensional (2D) *in vitro* methods often fail to adequately replicate tumor cell interactions with the TME and to properly assess drug activity, here, to evaluate the effects of maraviroc, we developed and used a three-dimensional (3D) multicellular heterospheroid model³³ formed by tumor cells, monocytes and MSCs. By using 3D heterospheroids, in which tumor cells and different types of normal cells interact and organize their positions, we found that interactions between MSCs, monocytes and cHL cells increased the overall secretion of CCL5. Maraviroc decreased heterospheroid self-assembling, cell viability and cHL clonogenic growth ability, suggesting that it may counteract TME formation and, as a consequence, its protective effects. In a mouse xenograft model, maraviroc decreased the *in vivo* growth of both L-540 and L-428 cells by more than 50% and reduced monocyte infiltration without apparent toxicity to the animals. These findings confirm that CCR5 signaling contributes to determining the fate of cHL tumor cells.

Recently, Jiao et al.⁴² demonstrated that maraviroc dramatically enhanced cell killing of CCR5+ breast cancer cells by the DNA-damaging chemotherapeutic agent doxorubicin. Here, maraviroc synergized not only with doxorubicin, but also with brentuximab vedotin. These results suggest that CCR5 inhibitors, by enhancing the activity of other drugs, may allow a dose reduction of the two chemotherapeutic agents already in use in cHL patients.

As shown here, CCL5 expression levels positively correlated with CD68 levels in cHL tissues, and patients with high CCL5 levels had lower progression-free survival than patients with low or medium expression of this chemokine; this difference may be due to the higher level of macrophages recruited and then reprogrammed by cHL cells towards immunosuppressive tumor-associated PD-L1-positive macrophages as well as by enhanced tumor growth by CCL5.

In conclusion, in light of the significant reduction of tumor mass obtained with maraviroc alone, its low toxicity and ability to inhibit monocyte infiltration, our results provide the rationale for its clinical assessment in cHL, as a single agent or in combination therapy.

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Table 1. Combination index (CI) values for L-1236 and HDLM-2 cell lines treated with maraviroc (MVC) (first column) and with doxorubicin (Doxo), brentuximab vedotin (BV), cisplatin (CDDP), gemcitabine (GCB) or vinorelbine (VRB) for 72 h.

	MVC (μ M)	Doxo (ng/mL)	CI	BV (μ g/mL)	CI	CDDP (μ M)	CI	GCB (nM)	CI	VRB (ng/mL)	CI
L-1236	25	6.0	0.397	3.8	0.253	0.13	0.606	0.15	0.662	0.13	0.849
	50	12.5	0.594	7.5	0.357	0.25	0.955	0.30	0.912	0.25	0.946
	100	*25.0	0.628	*15.0	0.495	*0.50	1.152	*0.60	1.302	*0.50	1.051
HDLM-2	25	3.1	0.512	75	0.373	0.31	0.571	0.80	0.699	0.25	0.818
	50	6.2	0.526	150	0.478	0.63	0.706	1.60	1.054	0.50	0.887
	100	*12.5	0.753	*300	0.655	*1.25	1.169	*3.20	1.173	*1.00	1.045

Cell viability was determined by trypan blue dye exclusion. Combination indexes were calculated using CalcuSyn software.

* Indicates the IC₅₀ for each drug.

Figure legends

Figure 1. Maraviroc inhibits crosstalk between cHL cells and both MSCs and monocytes.

(A) Percentages of BM-MSCs and AT-MSCs that migrated (in 5 h) through a fibronectin-coated Boyden chamber towards conditioned medium (CM) from L-1236 or KM-H2 cells, in the presence of increasing concentrations of maraviroc (MSCs were treated for 1 h prior to migration). **(B)** Effect of a neutralizing anti-CCL5 antibody (5 µg/mL) in cHL conditioned medium on MSC migration. Transmigrated cells were revealed using a computer-interfaced GeniusPlus microplate reader (Tecan). Results are the mean and SD of three replicate wells from three independent experiments. **(C)** BM-MSCs, AT-MSCs and HL-MSCs (100 cells/well; 24-well plates) were cultured in RPMI-1640 medium containing 10% cHL CM. After 9 days, cells were fixed with methanol and stained with crystal violet. **(D)** BM-MSCs (500 cells/well; 96-well plates) were cultured in RPMI-1640 medium containing 20% cHL CM, with or without a neutralizing anti-FGF2 (1 µg/ml), anti-TGFβ1 (2 µg/mL) or anti-TNFα (0.5 µg/mL) antibody. After 9 days, growth was evaluated using the MTT assay. Results are the mean and SD of three replicate wells from three independent experiments. **(E)** BM-MSCs were cultured for 6 days with 20% CM from L-1236, L-428, KM-H2, HDLM-2, and L-540 cells. Then, the medium was changed with fresh medium, and 3 days later MSC CM was recovered and quantified for CCL5 by ELISA. All samples were tested in triplicate; conditioned media from three different experiments were evaluated. **(F)** BM-MSCs were cultured for 6 days with 20% CM (from KM-H2 or HDLM-2 cells) in the presence or absence of a neutralizing anti-TNFα antibody (0.5 µg/mL). Then, the medium was changed and after 3 days CCL5 was quantified by ELISA. All samples were run in triplicate; conditioned media from three different experiments were evaluated. **(G)** Clonogenic growth. L-1236 (10^3 /mL), HDLM-2 (5×10^2 /mL), L-540

(2.5×10^2 /mL) cells were cultured in methylcellulose-containing medium in the absence or presence of 5% E-BM-MSC CM and with increasing concentrations of maraviroc. After 14 days of incubation, plates were observed under phase contrast microscopy and aggregates with 40 cells or more were scored as colonies (8 replicate wells). Each experiment was done in triplicate; conditioned media from three different experiments were evaluated. **(H)** Percentage of CD14⁺ monocytes that migrated (in 1 h) through fibronectin-coated Boyden chambers towards medium (RPMI-1640 plus 10% FCS) or E-BM-MSC CM. Prior to migration towards E-BM-MSC CM, monocytes were pretreated with maraviroc (0.1-100 μ M) for 1 h. Results are means and SD of transmigrated monocytes for three different experiments.

Figure 2. cHL cells induce monocyte migration and proliferation.

(A) Percentages of monocytes that migrated (in 1 h) through fibronectin-coated Boyden chambers towards conditioned medium (CM) from L-1236 and L-428 cells, in the presence of increasing concentrations of maraviroc (monocytes were treated for 1 h prior to migration). **(B)** Effect of a neutralizing anti-CCL5 antibody (5 μ g/mL) in cHL conditioned medium on monocyte migration. Results are means and SD of three replicate wells from three independent experiments. **(C)** cHL cells (2×10^5 cells/mL) were cultured for 3 days before CM was collected and tested for M-CSF by ELISA. All samples were run in triplicate; conditioned media from three different experiments were evaluated. **(D)** Monocytes (2.0×10^4 cells/well; 96-well flat-bottomed plates) were exposed to increasing concentrations (percentage, v/v) of cHL CM. After 9 days, monocyte growth was evaluated using the MTT assay. Results are mean and SD of three experiments. **(E)** Clonogenic growth in methylcellulose-containing medium. L-1236 (10^3 /mL), HDLM-2 (5×10^2 /mL), L-540 (2.5×10^2 /mL) cells were cultured in

the absence or presence of 5% (v/v) E-monocyte CM, with increasing concentrations of maraviroc. After 14 days of incubation, plates were observed under phase contrast microscopy and aggregates with 40 cells or more were scored as colonies (8 replicate wells). Each experiment was done in triplicate; conditioned media from three different experiments were evaluated.

Figure 3. Monocyte conversion towards immunosuppressive E-monocytes by cHL cells.

(A) Purified CD14⁺ monocytes were cultured for 6 days in the absence or presence of 20% conditioned medium (CM) from L-1236, L-428, and L-540 cells (to convert monocytes into E-monocytes), then washed and cultured for another 72 h in fresh medium. This E-monocyte CM was recovered for IL-10, CCL17, and TGF β ELISAs. Results are means and SD of three independent experiments. **(B)** Representative flow cytometric histograms showing surface expression of CD206 and PD-L1 and intracellular expression of IDO in monocytes (control) and E-monocytes (treated with cHL CM). **(C)** E-monocyte CM was tested for immunosuppressive activity. Phytohemagglutinin (PHA)-activated lymphocytes were treated with increasing concentrations (percentage, v/v) of monocyte CM and E-monocyte CM. After 72 h, growth was assayed using the Cell Proliferation ELISA, BrdU. All samples were tested in triplicate; conditioned media from three different experiments were evaluated.

Figure 4. Maraviroc slows tumor cell growth and inhibits heterospheroid formation.

(A) cHL cells (2×10^5 cells/ml) were cultured with increasing concentrations of maraviroc for 72 h, and viable cells were counted by trypan blue dye exclusion. Results are means and SD of three replicate wells from three independent experiments. **(B)** Percentages of cHL cells in the various cell cycle phases after a 24 h treatment with maraviroc (100 μ M). Results are means and SD of at least three experiments. **(C)** Representative image of 3D heterospheroids generated by plating HDLM-2

cells (stained green with CFSE), MSCs (red with fluorescent Dil), and monocytes (blue with DiD) under non-adherent conditions (poly-HEMA-coated wells). **(D)** L-1236 or HDLM-2 cells, HL-MSCs and monocytes were cultured in RPMI 1640 medium plus 1% FCS, alone or in combination under non-adherent conditions. After 4 days, conditioned medium was collected for CCL5 ELISAs; three different experiments were evaluated. **(E)** Heterospheroids generated by co-cultivation of cHL cells (L-1236, HDLM-2, or L-540 cells) with HL-MSCs and monocytes under non-adherent conditions in the presence or absence of maraviroc (100 μ M) and photographed after 24 h using an inverted microscope (Eclipse TS/100, Nikon). **(F)** Heterospheroids (cHL + HL-MSCs + monocytes) were cultured with and without maraviroc. After 48 h, viable cells were evaluated using PrestoBlue Cell Viability Reagent. Relative fluorescence units (RFU). Values are means and SD of three experiments.

Figure 5. Maraviroc reduces cHL xenograft growth and TAM infiltration.

(A-D) Xenografts in nude mice inoculated with L-540 cells (20×10^6 cells/animal) and treated every day with an intraperitoneal injection of 10 mg/kg maraviroc (n = 5) or vehicle (n = 5). **(A)** Xenograft tumor growth. **(B)** Body weights of xenografted mice. **(C)** Quantification of CD68+ staining in immunofluorescent cryosections using Volocity software provided by PerkinElmer (arbitrary units). Data are means and SD. **(D)** Immunofluorescent photomicrographs of CD68+ staining in tumor cryosections from maraviroc-treated and untreated xenografted mice. Nuclei were stained with TO-PRO-3 dye. Representative images were acquired using a confocal microscope (Leica DM IRE2). **(E-H)** Xenografts in NSG mice inoculated with L-428 cells (10×10^6 cells/animal) and treated every other day with an intraperitoneal injection of 10 mg/kg maraviroc (n = 5) or vehicle (n = 5). **(E)** Xenograft tumor growth. **(F)** Body weights of xenografted mice. **(G)** Quantification of CD68+ staining in

immunofluorescent cryosections using Volocity software (arbitrary units). Data are means and SD. **(H)** Immunofluorescent photomicrographs of CD68+ staining in tumor cryosections from maraviroc-treated and untreated xenografted mice. Nuclei were stained with TO-PRO-3 dye. Representative images were acquired using a confocal microscope (Leica DM IRE2).

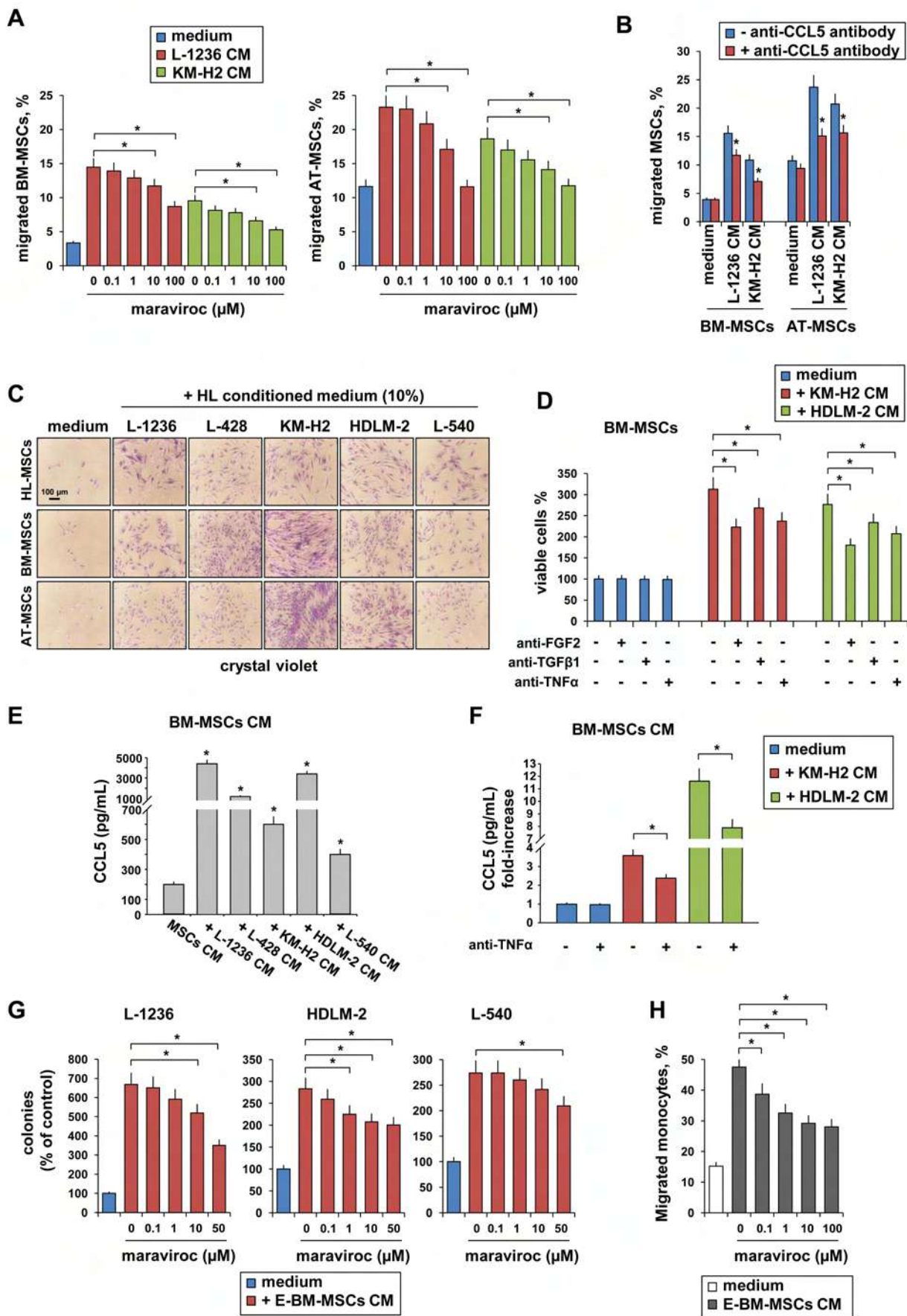
Figure 6. CCL5 expression positively correlates with CD68 expression in human cHL tissues and with lower progression-free survival in cHL patients.

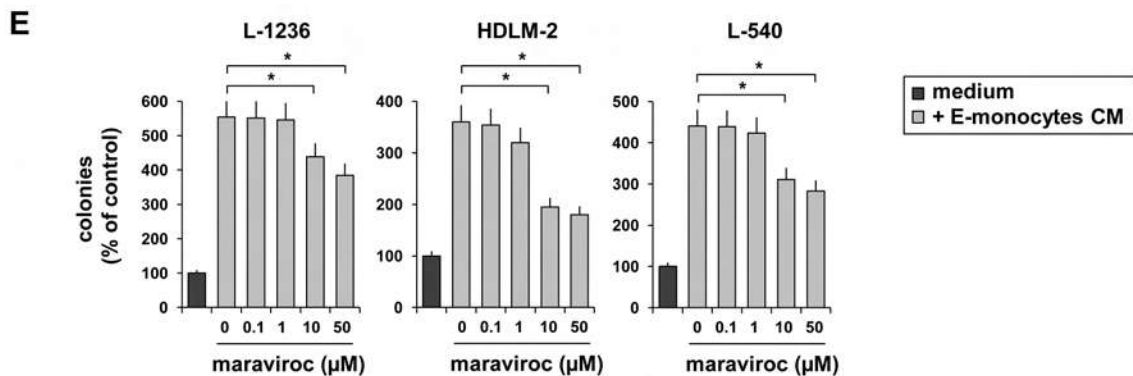
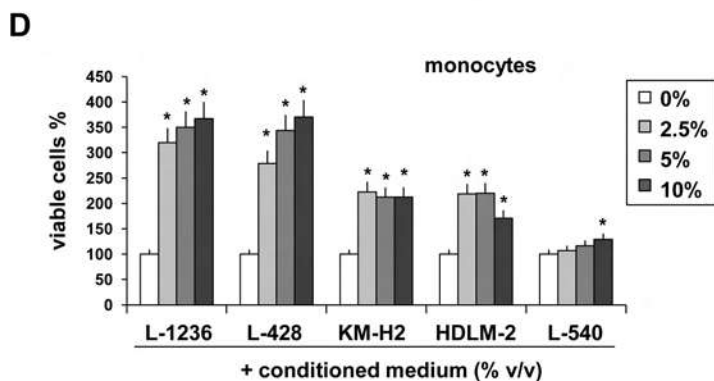
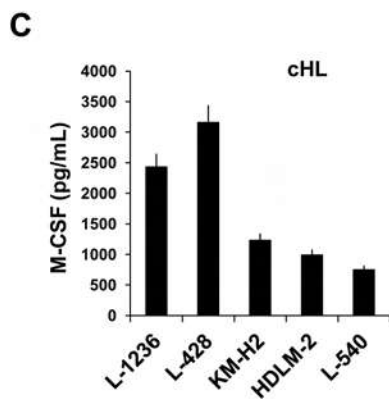
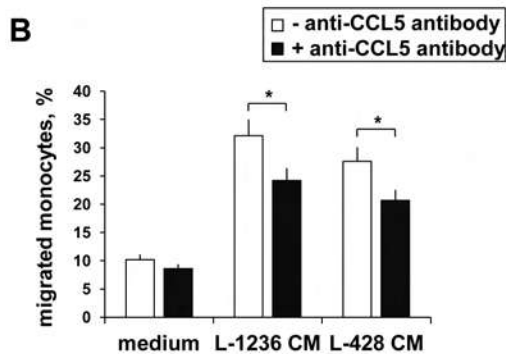
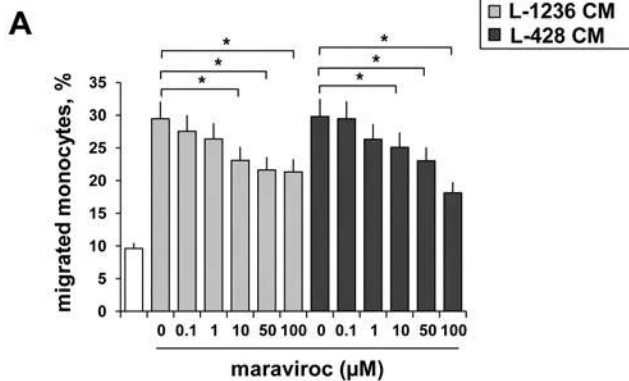
(A) Representative photomicrographs of cHL tissues stained for CCL5 and CD68 (marker of macrophages/monocytes). *Top row*, a case with low expression; *bottom row*, a case with high expression. **(B)** Correlation of expression levels of CCL5 and CD68 in 65 cHL patients. Spearman $r=0.251$; $P=0.0487$. **(C)** Kaplan-Meier survival plot for 5-year progression-free survival in cHL patients, subdivided according to the percentage of CCL5 positivity (low, 0%-10%; medium, 11%-20%; high, 21%-100%). Patients with high CCL5 expression had worse survival ($P=0.0072$, HR=0.015 vs. low-medium levels).

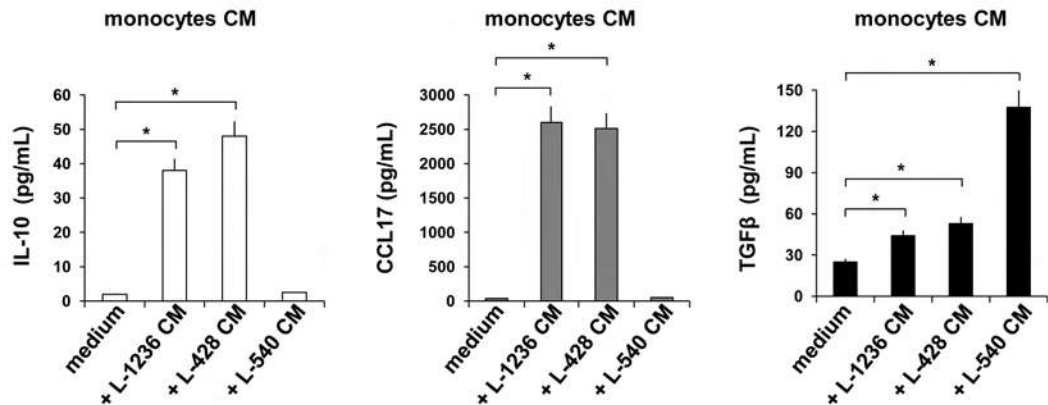
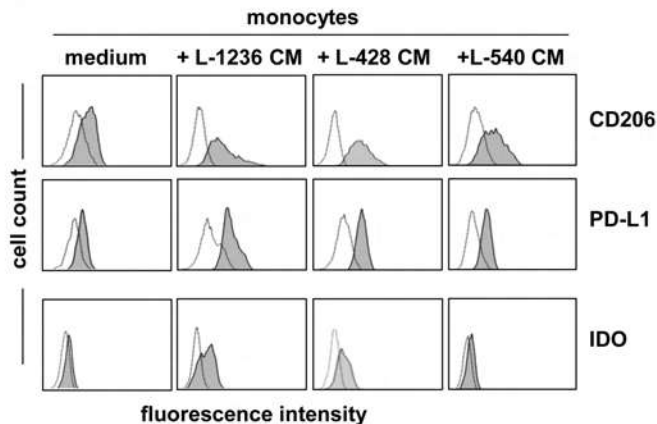
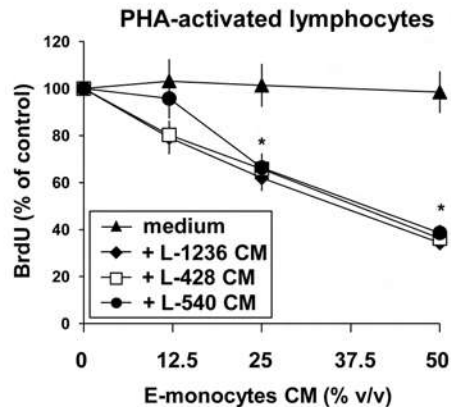
Figure 7. Proposed mechanism for the inhibitory effects of the CCR5 antagonist maraviroc on TME formation and cHL tumor growth.

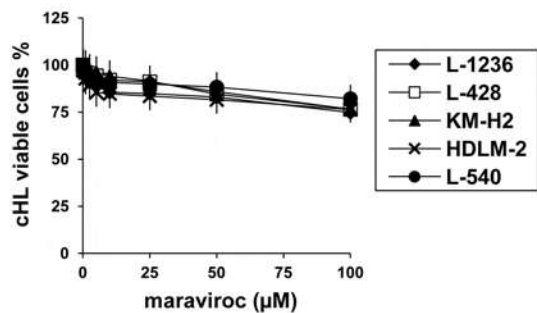
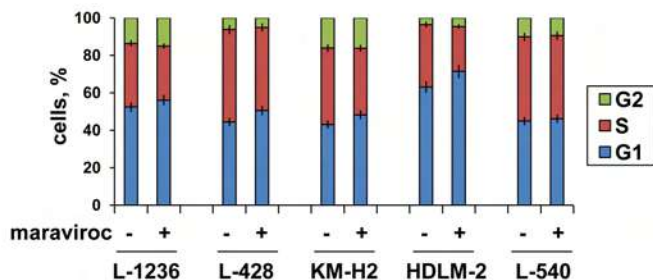
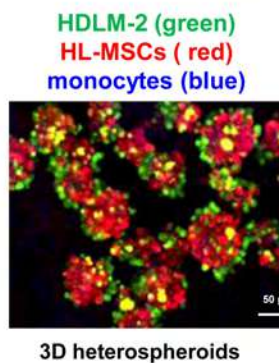
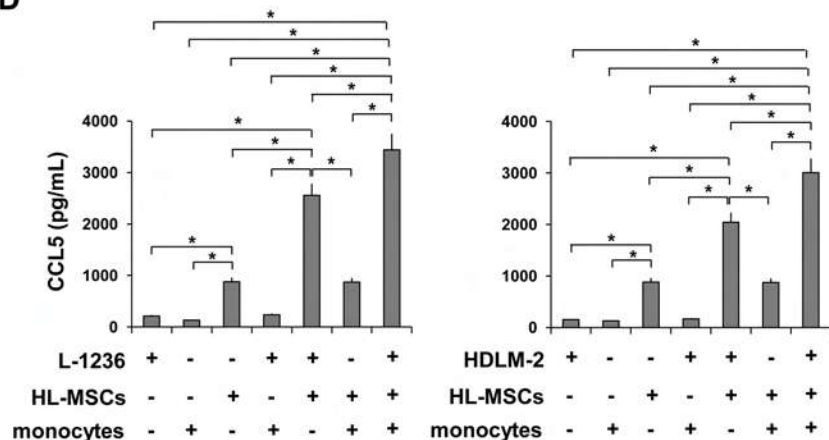
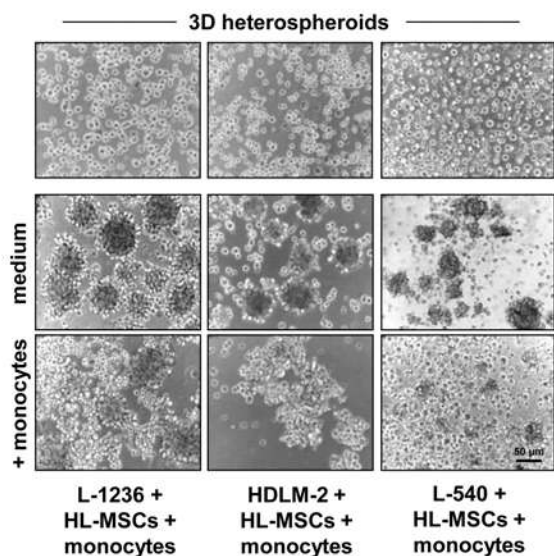
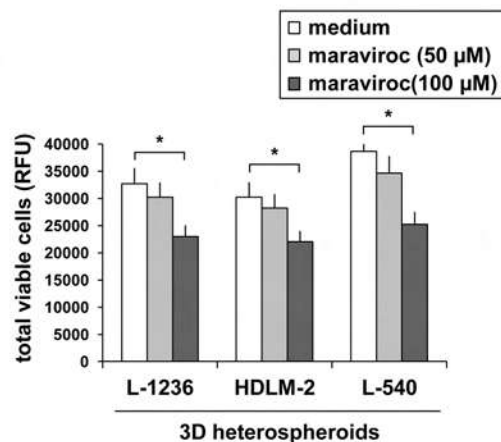
(1) Maraviroc, a CCR5 antagonist, inhibits the recruitment of monocytes and MSCs by cHL cells (Hodgkin and Reed/Sternberg, HRS). **(2)** The tumor “education” of MSCs cells (E-MSCs) induces the secretion of CCL5. **(3)** Maraviroc inhibits the recruitment of monocytes by E-MSCs secreting CCL5. **(4)** Maraviroc decreases the cHL cell growth-promoting effects of CCR5 ligands secreted by tumor educated-monocytes (E-monocytes) and E-MSCs (CCL5+). **(5)** Maraviroc inhibits the growth of cHL cells (autocrine growth). **(6)** cHL cells reprogram monocytes, and E-monocyte increases the expression of

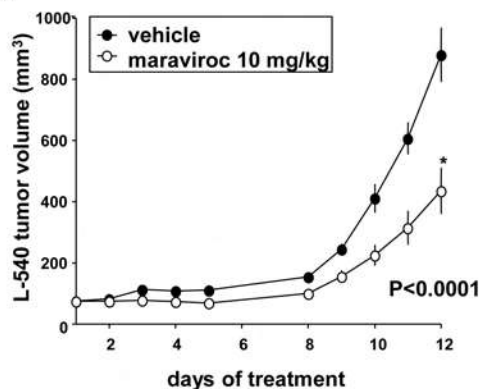
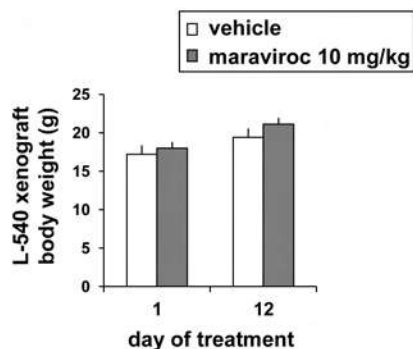
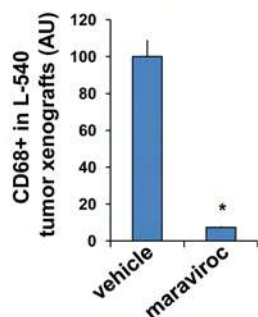
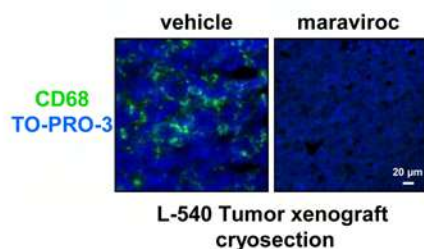
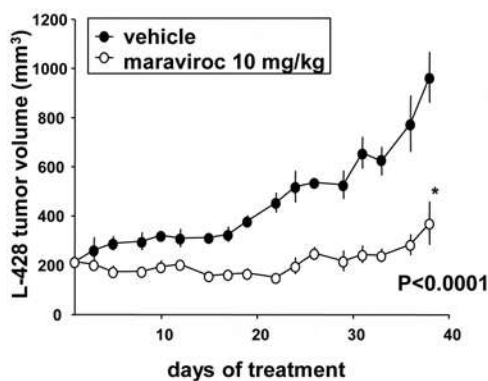
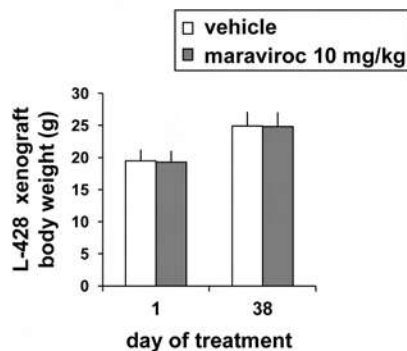
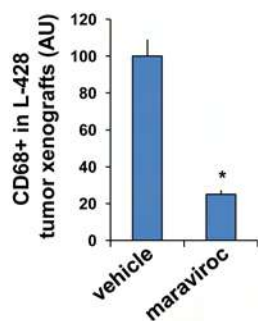
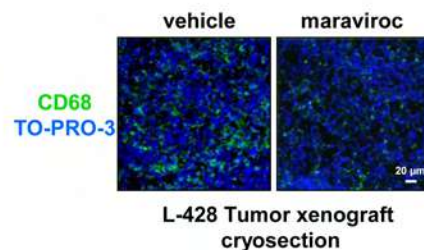
CD206, PD-L1 and IDO (immunosuppressive phenotype). TME, tumor microenvironment. MVC, maraviroc.





A**B****C**

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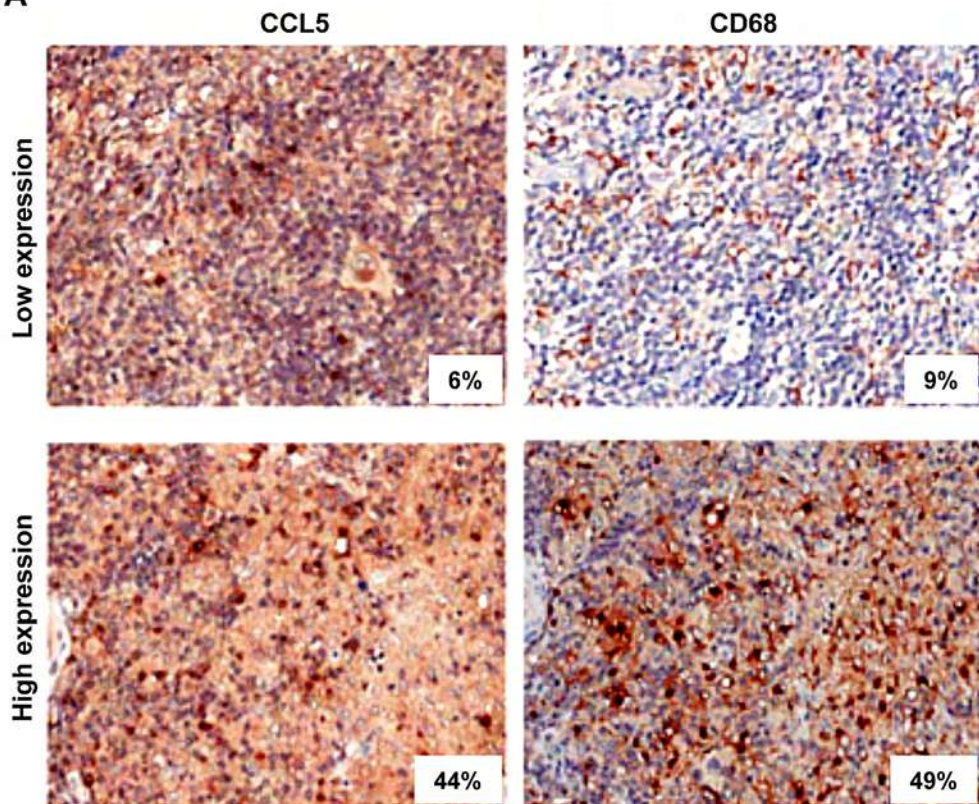
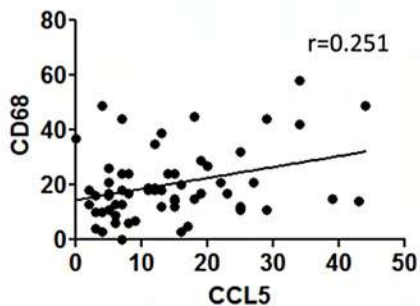
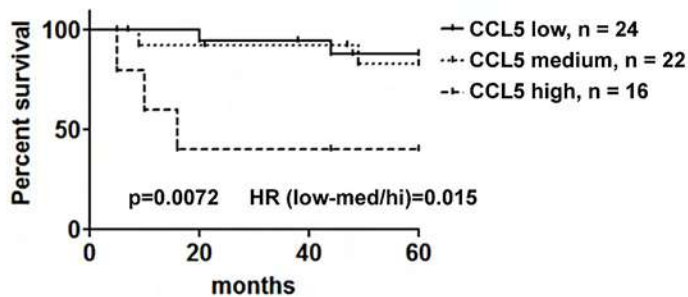
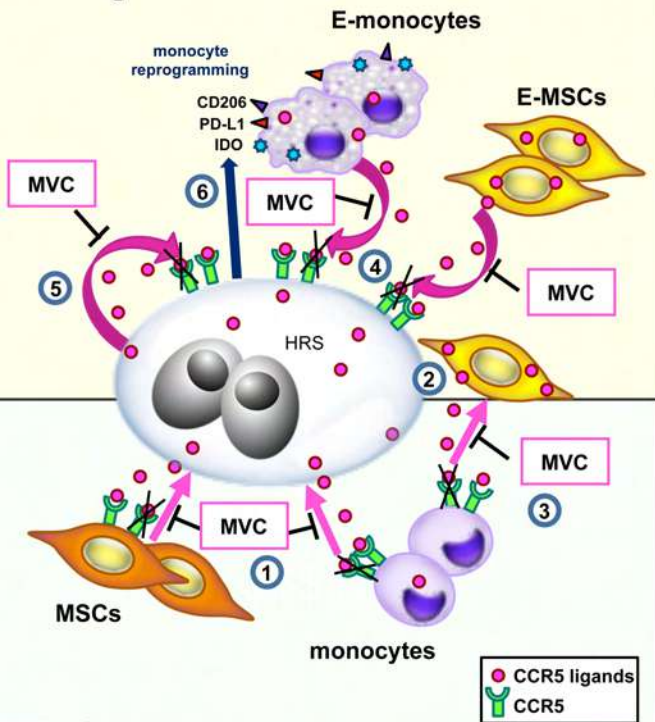
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Figure 7

Tumor growth



TME formation

Supplementary methods

Drugs

The following drugs used in the study were surplus samples obtained from the clinical pharmacy of CRO Aviano: doxorubicin (Pfizer), brentuximab vedotin (Takeda), cisplatin (Teva), gemcitabine (Actavis), and vinorelbine (Pierre Fabre).

Preparation of primary MSCs from cHL-involved lymph nodes.

Three separate frozen lymph node preparations obtained from a patient affected by a nodular sclerosis variant of HL of non-T, non-B phenotype (CD15+, CD30+, CD40+, CLA–, CD20–, CD79a–, CD3–, ALK–) were employed as sources of MSCs from HD-involved lymph nodes (HL-MSCs)¹. Single cell suspension obtained by mechanical dissociation of lymph node fragments was cultured in Mesenchymal-Stem-Cell Growth Medium Bulletkit (Lonza) to avoid differentiation. After non-adherent cells had been removed, adherent cells (MSCs)² were cultured in Mesenchymal-Stem-Cell Growth Medium Bulletkit.

Fluorescence-assisted transmigration invasion and motility assay

Cells (MSCs: 50,000 cells/insert; purified CD14+ monocytes: 200,000/insert) were tagged with the lipophilic CellTracker™ CM-Dil Dye (Thermo Fisher Scientific) as described.³ Cells were then seeded in 150 µl serum-free medium in the upper side of fibronectin-coated (20 µg/mL) Boyden chambers inserted into the wells of 24-well cell culture plates. Below the Boyden chambers, the wells contained 700 µL of RPMI-1640 plus 10% FCS (control medium) or conditioned medium from cHL cell lines (cHL CM) or from tumor-educated MSCs (E-MSC CM) for testing as a chemoattractant. In some

experiments, the CCR5 antagonist maraviroc (0.1 to 100 μ M final concentration) was added to cells, or a mouse anti-human CCL5 antibody (5 μ g/mL; clone 21418, R&D Systems) was added to the chemoattractant 1 h prior to migration. Transmigrated cells were revealed using a computer-interfaced GeniusPlus microplate reader (Tecan).

Proliferation, clonogenic growth and senescence assays

In some experiments, MSCs were plated in culture medium or in cHL conditioned medium (500 cells/well) containing neutralizing anti-human FGF2 (AF233, R&D Systems; 1 μ g/mL), anti-human TGF β 1 (TB21, GeneTex; 2 μ g/mL), or anti-TNF α (AF-210, R&D Systems; 0.5 μ g/mL) antibodies or no antibody. Half the volume of culture medium was replaced every 2 days. After 9 days, growth was evaluated using the MTT assay. In other experiments, MSCs (100 cells/well, 24-well plates) or monocytes (2.0×10^4 cells/well, 96-well plates) were cultured for 9 days in RPMI-1640 with 10% cHL conditioned medium (final concentration of FCS, 1%) or in RPMI-1640 containing 1% FCS (medium control) and, then fixed with methanol and stained with crystal violet.

cHL cells (2.0×10^5 cells/mL) were cultured for 72 h with increasing concentrations of maraviroc; then viable cells were counted using the trypan blue dye exclusion method. Clonogenic growth was assessed as previously described.⁴ Briefly, L-1236 (10^3 /mL), HDLM-2 (5×10^2 /mL), and L-540 (2.5×10^2 /mL) cells were suspended in 1 mL of RPMI-1640 medium containing 15% FCS and 0.8% methylcellulose (Methocel, Fluka) in the presence of E-MSC conditioned medium or E-monocyte conditioned medium, with or without maraviroc (50 μ M) or a neutralizing anti-CCL5 antibody (5 μ g/mL; clone 21418, R&D Systems) and seeded in 100 μ L aliquots (8 replicates) in 96-well flat-

bottomed microplates. After 14 days of incubation, plates were observed under phase-contrast microscopy, and aggregates with 40 cells were scored as colonies.

To study senescence, MSCs (1000/well) were cultured in serum-free medium for 15 days, with and without cHL conditioned medium (10%, v/v). β -galactosidase activity was evaluated with the Senescence Cells Histochemical staining kit (Sigma-Aldrich).

Flow cytometry

Surface expression of CD206 and PD-L1 was evaluated using PE-Cy5 Mouse Anti-Human CD206 and FITC Mouse Anti-Human PD-L1/CD274 (clone MIH1) antibodies (Becton Dickinson). Intracellular IDO expression was examined using a rabbit anti-IDO mAb (clone D5j4ETM; Cell Signaling Technology) followed by phycoerythrin (PE)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Annexin-V (Becton-Dickinson Pharmingen) binding, staining with propidium iodide (PI) and 7-aminoactinomycin D (7-AAD; Becton-Dickinson), and detection of activated caspase 3/7 with CaspaTag Caspase 3/7 (Chemicon International, Milan, Italy) were evaluated as previously described.⁵ CCR5 expression was analyzed using the anti-CCR5 mAb (clone 45531; R&D Systems). KG-1 cells (human acute myeloid leukemia) were used as a negative control for CCR5 expression. Cell cycle progression after a 24 h treatment with maraviroc (100 μ M) was evaluated by propidium iodide staining. These analyses were done on a FACScan flow cytometer (Becton Dickinson), using CellQuest software. Cell cycle was analyzed using Mod-Fit LT software (Verity Software House, Topsham (ME), USA).

ELISAs

Cytokines/chemokines were quantified using commercially available ELISA kits for CCL3, CCL4 and CCL5, M-CSF, IL-10 and CCL17/TARC (all from Immunological Sciences) and TGF- β 1 (Invitrogen).

Allogeneic lymphocytes treated with E-monocyte conditioned medium

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor blood by density gradient centrifugation on Ficoll-PaqueTM PLUS (GE Healthcare), and subsequently incubated with Pharm Lyse Lysing Buffer (BD Biosciences) for 5 min at 4 °C to minimize the contamination with red blood cells. Monocyte depletion was carried out by plastic adherence (2 h).

Lymphocytes (1×10^5 cells/well in 96-well dishes) were prestimulated for 1 h with 2 μ g/mL phytohemagglutinin (Sigma). Then, they were cultured with increasing concentrations of E-monocyte conditioned medium. After 72 h, cell growth was measured using the Cell Proliferation ELISA, Brd-U (colorimetric) kit (Sigma).

Synergy

cHL cells (2×10^5 /mL) were cultured for 72 h with one or more drugs in combination, and viable cells were counted by trypan blue dye exclusion. Synergy was determined by calculating the combination index (CI)⁶ using CalcuSyn software (Biosoft, Ferguson, MO, USA). The combined drug effect was calculated using the diagonal constant ratio combination. A CI value ranging from 0.9 to 1.1 indicates an additive effect between two drugs. CI values <0.9 indicate synergy, the lower the value the stronger the synergy. On the contrary, CI values >1.1 indicate antagonism.

Evaluation of heterospheroid formation by fluorescence microscopy and tumor cell purification

To monitor heterospheroid formation, cells were stained before they were placed in cocultivation in non-adherent conditions. For this purpose, MSCs were stained with the fluorescent dye Dil (red),

CD14⁺ monocytes with DiD (blue) and HDLM-2 cells with CellTrace CFSE Cell Proliferation Kit (green; all dyes from Thermo Fisher Scientific). After 24 h, images were acquired using a confocal microscope (Leica DM IRE2). To isolate single tumor cells, heterospheroids were trypsinized and cHL cells (CD30⁺) were recovered using CD30 microbeads (Miltenyi Biotec). Then viable cells were counted, analyzed for colony growth and cell cycle progression.

Tumor xenograft: immunofluorescence and survival analysis

Mice were killed (L-540 xenografts after 12 days of treatment; L-428 xenografts after 38 days of treatment) and tumors were excised and frozen in the Killik embedding medium (Bio Optica, Milano, Italy). Frozen tumor sections were stained with rat anti-mouse CD68 antibody (MCA1957GA, Bio-Rad), rabbit anti-human CD30 antibody (EPR4102, ABCAM) and TO-PRO-3 (Invitrogen). Specific Alexa Fluor-conjugated secondary antibodies were applied. Fluorescence intensity (CD68⁺) was quantified using Volocity software (PerkinElmer).

To study survival of mice bearing L-540 xenografts, in adherence of rules of the CRO Aviano committee on animal experimentation (OBPA) and of the Italian Ministry of Health, we used a predefined cutoff volume of 800 mm³ as a surrogate for mortality.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v6 software. Student's *t* test was used to compare two groups, and one-way analysis of variance (ANOVA) was used for three or more groups; consecutive multiple comparisons were performed using Dunnett's or Tukey's test. Spearman's test was used to correlate CCL5 and CD68 expression. Survival was analyzed with Kaplan-Meier plots and log-rank test. Hazard ratios and 95% confidence intervals were calculated. **P*<0.05 indicated

statistical significance.

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Supplementary Table S1. Clinical characteristics of 65 cHL patients, with a median age of 31 years (range, 6-83 years).

Characteristic		Patients, n (%)
Sex	Male	39 (65)
	Female	21 (35)
cHL subtype	Nodular sclerosis	51 (7)
	Mixed cellularity	5 (8)
	Nodular lymphocyte predominant	8 (12)
	Not otherwise specified	1 (2)
Epstein-Barr virus	Positive	19 (29)
	Negative	46 (71)
Stage	I/II	23 (61)
	III/IV	15 (39)
Bulk	No	31 (82)
	Yes	7 (18)
B symptoms	No	22 (58%)
	Yes	16 (42%)
Treatment	Chemotherapy	16 (41)
	Combined modality	21 (54)
	Radiotherapy	2 (5)

Supplementary Table S2. Combination index (CI) values for L-1236 and HDLM-2 cell lines treated with maraviroc (MVC) (first column) and with doxorubicin (Doxo) or brentuximab vedotin (BV) for 72 h.

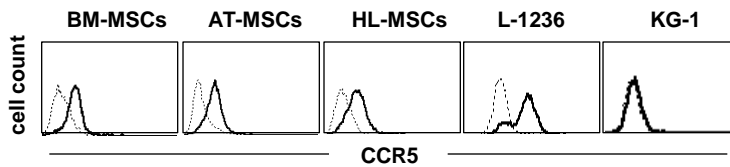
	MVC (μ M)	Doxo (ng/mL)	CI	BV (μ g/mL)	CI
L-1236	2.5	6.3	0.453	3.8	0.560
	5.0	12.5	0.714	7.5	0.421
	10.0	*25.0	0.623	*15.0	0.363
HDLM-2	2.5	3.1	0.408	75	0.509
	5.0	6.3	0.473	150	0.338
	10.0	*12.5	0.675	*300	0.441
L-1236	0.25	6.3	0.807	3.8	0.951
	0.50	12.5	1.130	7.5	0.852
	1.00	*25.0	1.125	*15.0	1.232
HDLM-2	0.25	3.1	1.105	75	1.332
	0.50	6.3	1.146	150	0.726
	1.00	*12.5	1.211	*300	0.948

Cell viability was determined by trypan blue dye exclusion. Combination indexes were calculated using CalcuSyn software.

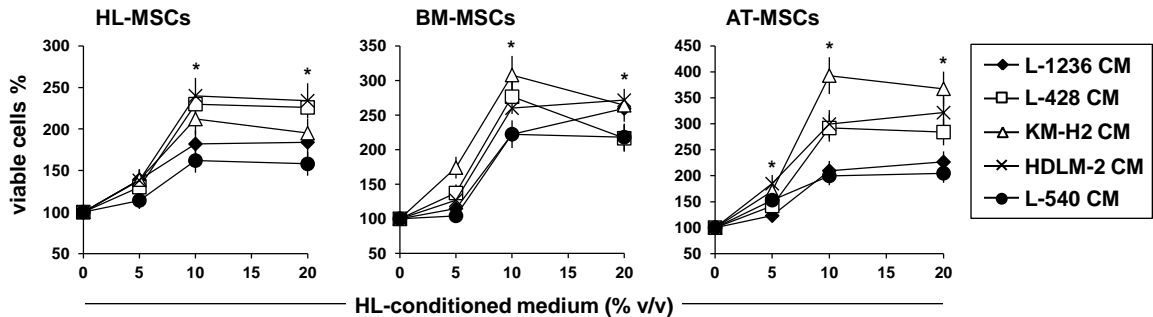
* indicates the IC₅₀ for each drug.

Supplementary Figure S1

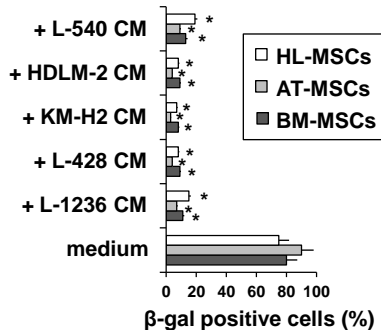
S1A



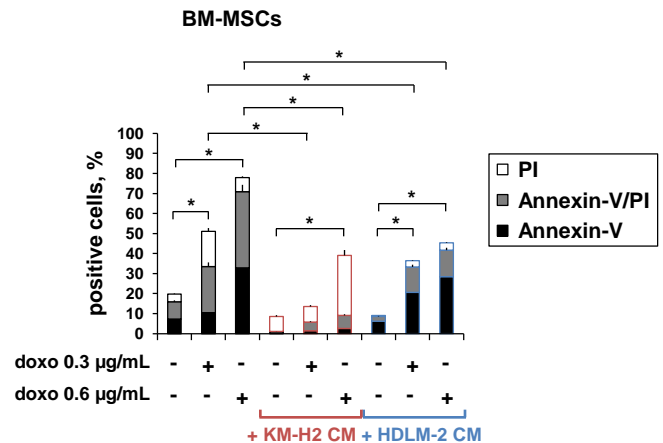
S1B



S1C

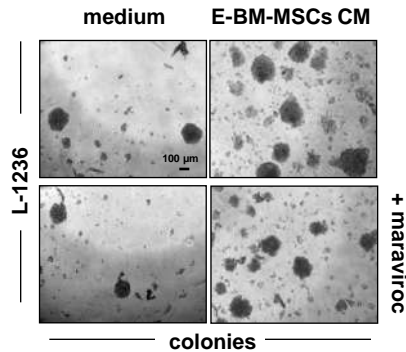


S1D

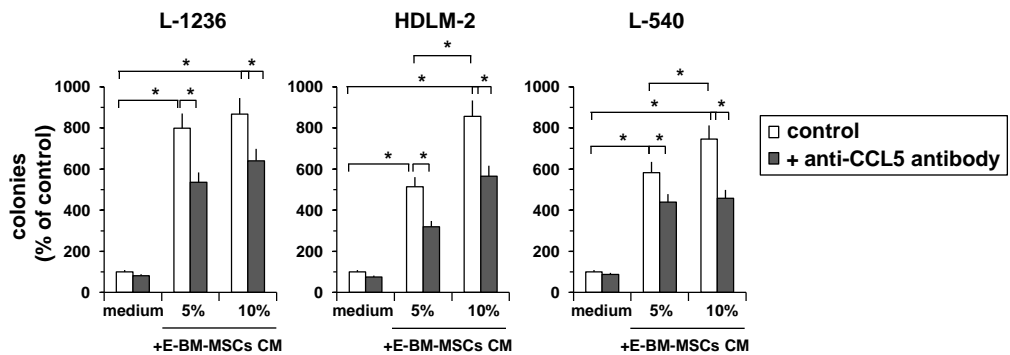


Supplementary Figure S1. CCR5 expression by MSCs and effects of cHL cell conditioned medium on MSC growth, senescence and doxorubicin cytotoxicity. (A) CCR5 expression was evaluated using the anti-CCR5 mAb (clone 45531; R&D Systems) in BM-MSCs, AT-MSCs, HL-MSCs, L-1236 cells and KG-1 cells (negative control). (B) BM-MSC, AT-MSC and HL-MSC (500/well) were cultured in 96-well plates in RPMI-1640 medium in increasing concentrations (percentage, v/v) of cHL conditioned medium (CM) that was replaced twice. After 6 days, growth was evaluated using the MTT assay. Results are means and SD of three replicate wells from three independent experiments. (C) Senescence-associated beta-galactosidase (β -gal) activity. Briefly, 1,000 MSCs were cultured in 24-well plates in RPMI-1640 medium in the absence or presence of 10% (v/v) cHL CM. After 15 days, β -gal-positive cells were counted. Three different experiments were evaluated. (D) BM-MSCs were treated with doxorubicin (doxo) (0.3 and 0.6 μ g/mL) in the presence or absence of 20% KM-H2 or HDLM-2 CM. After 6 days, cells were harvested, double stained with annexin-V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Bar charts show the percentages of stained cells. Values are means and SD of three experiments.

S2A

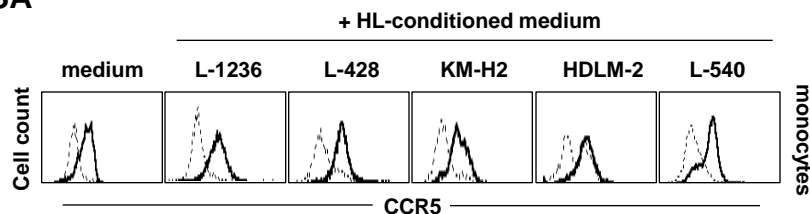


S2B

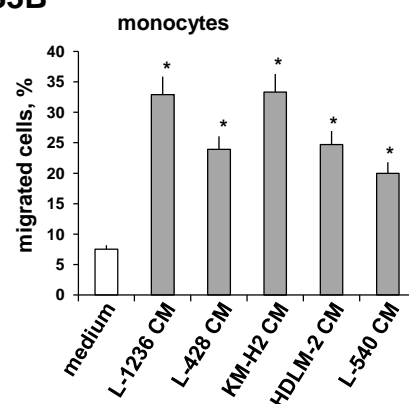


Supplementary Figure S2. Maraviroc and an anti-CCL5 antibody reduce the clonogenic growth of cHL cells enhanced by MSC CM. (A) Clonogenic growth. L-1236 cells ($10^3/\text{mL}$) were cultured in methylcellulose-containing medium in the presence or absence of E-BM-MSC conditioned medium (CM), with or without maraviroc ($50 \mu\text{M}$). After 14 days, colonies were photographed using an inverted microscope (Eclipse TS/100, Nikon). (B) L-1236 ($10^3/\text{mL}$), HDLM-2 ($5 \times 10^2/\text{mL}$), and L-540 ($2.5 \times 10^2/\text{mL}$) cells were cultured in methylcellulose medium in the presence or absence of E-BM-MSC CM, with or without a neutralizing anti-CCL5 antibody ($5 \mu\text{g}/\text{mL}$). Bar charts show the percentages of colonies normalized to control samples. Three different experiments were evaluated.

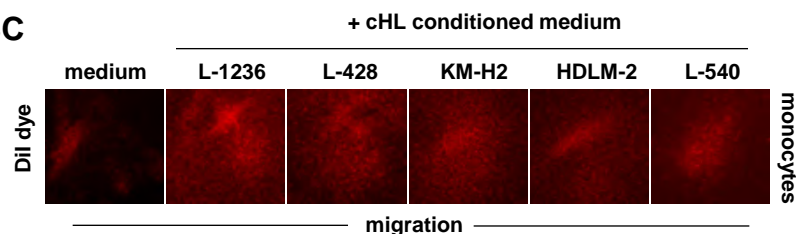
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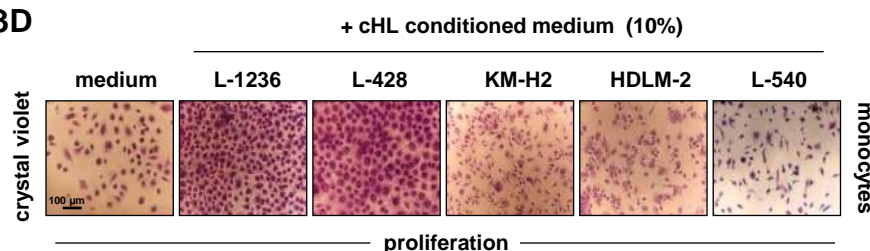
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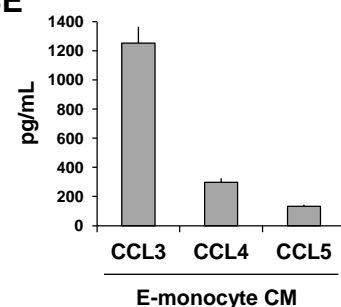
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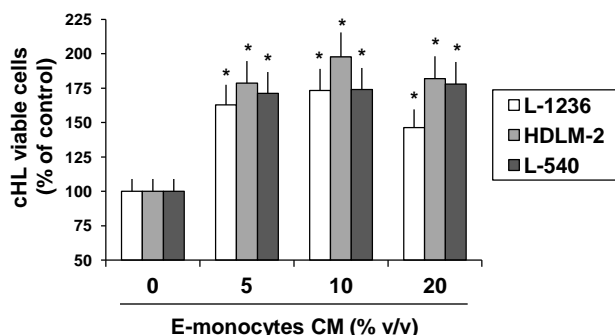
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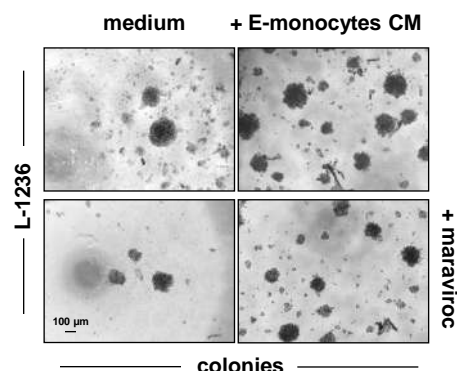
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S3F

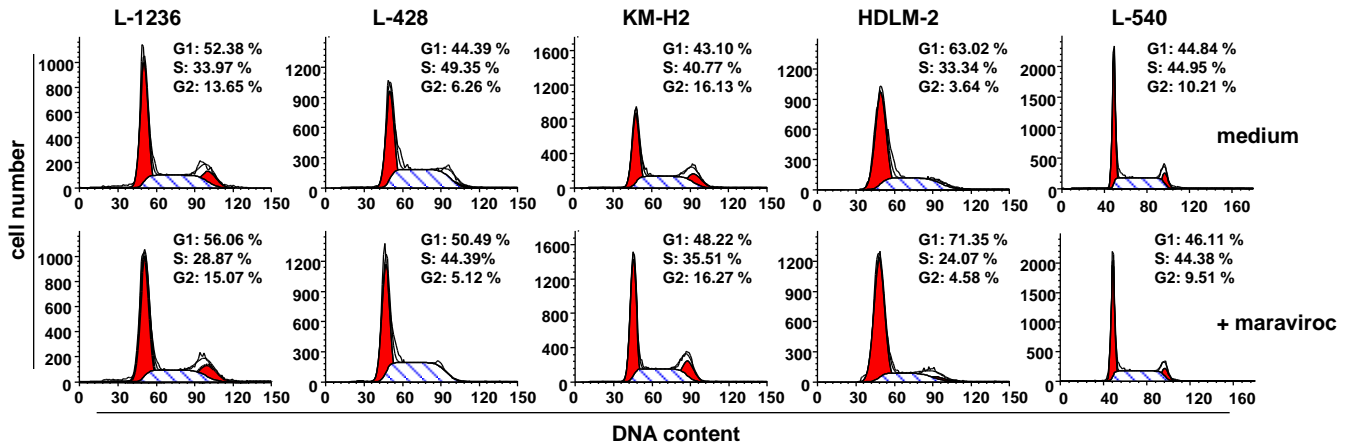


S3G

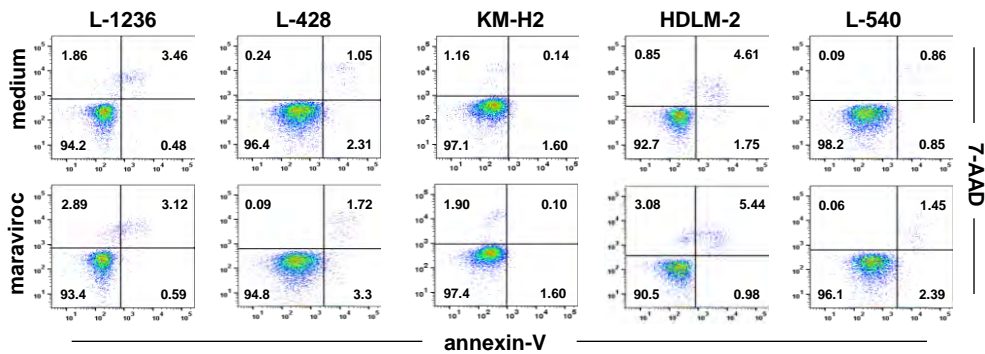


Supplementary Figure S3. Effects of cHL CM on monocyte migration, proliferation, CCR5-ligand secretion and of E-monocytes CM on cHL growth. (A) Purified CD14⁺ monocytes were cultured for 6 days with 20% CM from L-1236, L-428-, KM-H2, HDLM-2, and L-540 cells, inducing monocytes into becoming E-monocytes. CCR5 expression was evaluated by flow cytometry. **(B)** 1 h migration of human CD14⁺ monocytes across fibronectin-coated Boyden chambers towards cHL CM. CM from different experiments were evaluated. **(C)** Representative photomicrographs of Boyden chamber membranes showing transmigrated CD14⁺ monocytes stained with red fluorescent dye Dil. **(D)** Monocytes (2.0×10^4 /well) were cultured in 96-well flat-bottomed plates and exposed to increasing concentrations (%) of cHL CM that was replaced twice. After 6 days, monocytes were fixed with methanol and stained with crystal violet. **(E)** Monocytes were cultured with 20% L-1236 CM for 6 days. The medium was changed and, after another 3 days, E-monocyte CM was recovered. CCL3, CCL4 and CCL5 release was evaluated by ELISA. **(F)** HDLM-2, L-1236, and L-540 cells (2.5×10^3 /mL) were treated for 9 days with E-monocyte CM, replaced twice. Viable cells were evaluated by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega). Values are means and SD of three experiments. **(G)** Clonogenic growth in methylcellulose-containing medium. L-1236 cells were cultured in the presence of E-monocyte CM (5%) and treated or not with maraviroc (50 μM). Colonies were counted and L-1236 cells were photographed using an inverted microscope.

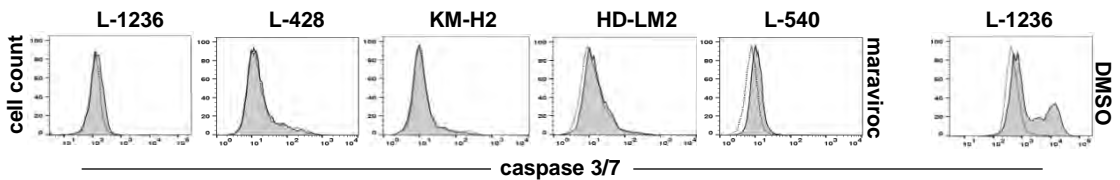
S4A



S4B

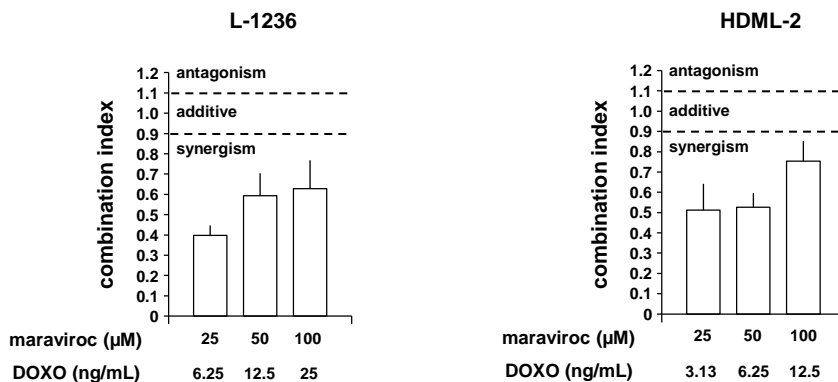


S4C

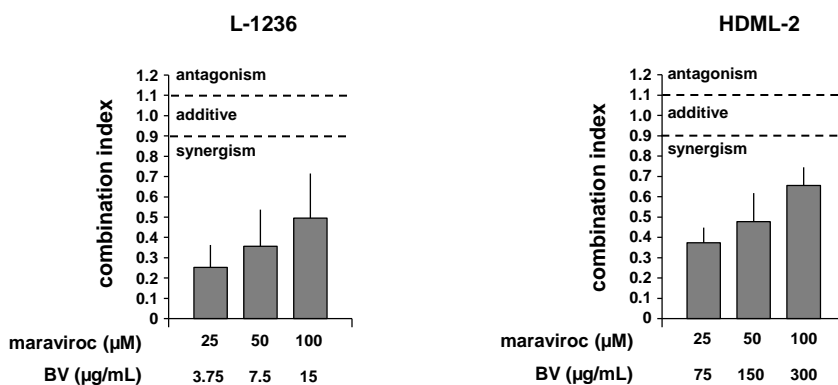


Supplementary Figure S4. Maraviroc slightly increases the percentage of cHL cells in G1 phase but does not induce apoptosis. (A) Representative cytofluorimetric histograms of the cell cycle progression. cHL cells (2.0×10^5) were treated for 24 h with maraviroc (100 μ M), and then stained with propidium iodide (PI) and analyzed by flow cytometry. **(B, C)** cHL cells were treated for 72 h with maraviroc (100 μ M) and **(B)** then doubly stained with annexin-V-FITC and 7-AAD or **(C)** resuspended in complete medium supplemented with CaspaTag Caspase 3/7 (Chemicon) for 1 h at 37°C to evaluate caspase 3/7 activation by flow cytometry. L-1236 cells treated with DMSO were used as positive control.

S5A



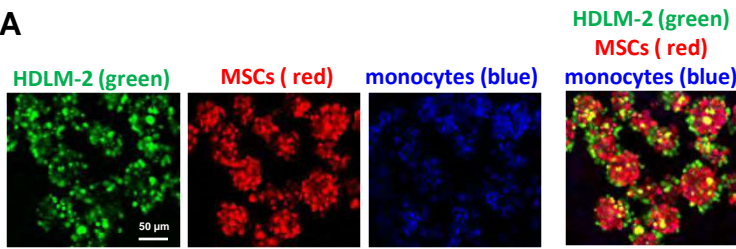
S5B



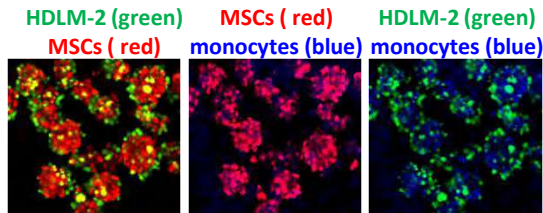
Supplementary Figure S5. Drug combination curves of maraviroc with doxorubicin or brentuximab vedotin. L-1236 and HDLM-2 cells were cultured with increasing concentrations of maraviroc and **(A)** doxorubicin (DOXO) or **(B)** brentuximab vedotin (BV), alone or in combination. Viable cells were evaluated by trypan blue dye exclusion after 72 h. Synergy was determined using CompuSyn software (CI, combination index). Values of bar charts are mean CI values and SD of three experiments each run in triplicate.

Supplementary Figure S6

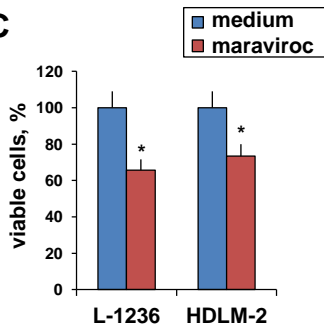
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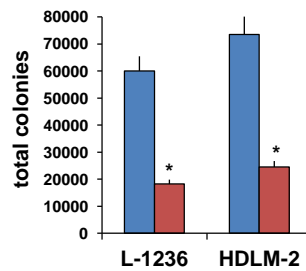
3D heterospheroids



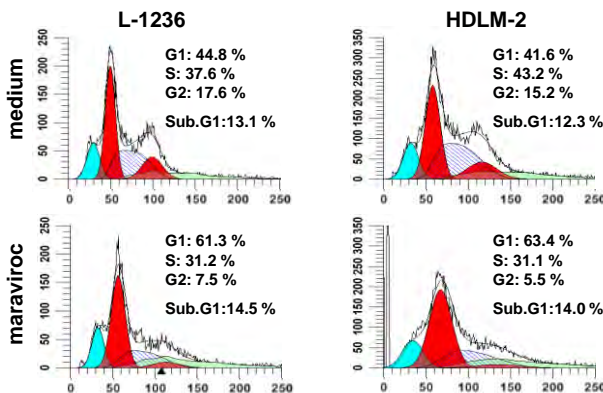
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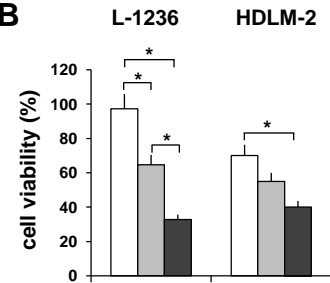
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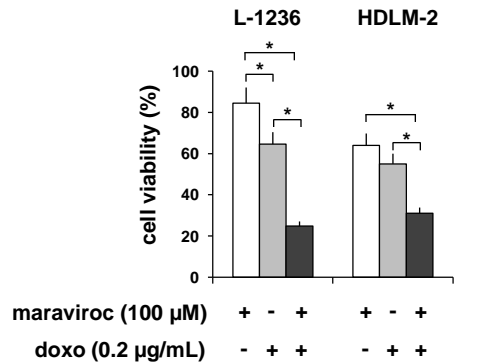
S6E



S6B



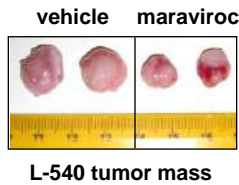
MVC (μM)	Doxo (μg/mL)	CI L-1236	CI HDLM-2
50	0.1	0.669	0.607
50	0.2	0.321	0.453
50	0.4	0.335	0.567



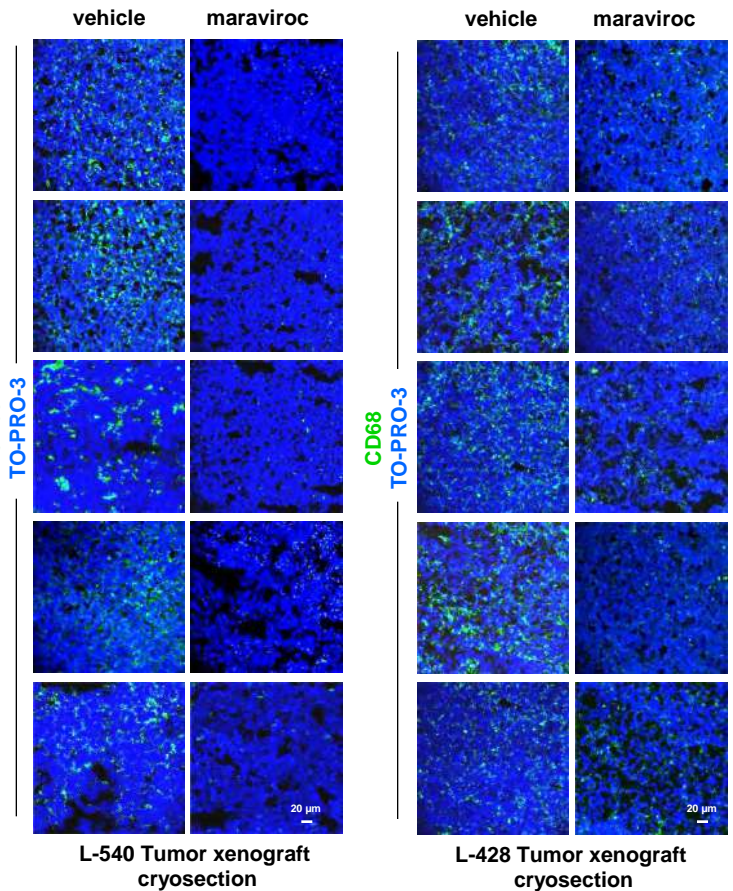
MVC (μM)	Doxo (μg/mL)	CI L-1236	CI HDLM-2
100	0.1	0.588	0.629
100	0.2	0.418	0.271
100	0.4	0.46	0.293

Supplementary Figure S6. Effects of maraviroc on heterospheroids. (A) 3D heterospheroids were generated by culturing HDLM-2 cells (stained green with CFSE), MSCs (red with fluorescent dye DiI), and monocytes (blue with DiD) under non-adherent conditions on poly-HEMA-coated wells. Photomicrographs were acquired using a confocal microscope (Leica DM IRE2). (B) Maraviroc-doxorubicin drug combination was assessed in heterospheroids generated with L-1236 cells, HL-MSCs and monocytes (2.0×10^3 cells/well; 96-well plates) in RPMI-1640 medium plus 1% FCS with increasing concentrations of doxorubicin (doxo) and either 50 μM (upper panel) or 100 μM (lower panel) maraviroc, alone or in combination. After 6 days, cell viability was evaluated using PrestoBlue Cell Viability kit. Values are means and SD of three experiments each run in triplicate. Synergy was determined using CompuSyn software (CI, combination index). CI values <0.9 indicate synergy. (C) Viable L-1236 or HDLM-2 cells isolated from heterospheroids (also containing HL-MSCs and monocytes) after a 48 h treatment with maraviroc (100 μM). Values are percentages normalized to untreated samples. (D) Total number of colonies formed in methylcellulose-containing medium from cHL cells recovered from heterospheroids. (E) Representative flow cytometric plots showing cell cycle progression of L-1236 or HDLM-2 cells purified from heterospheroids treated or not for 48 h with maraviroc (100 μM).

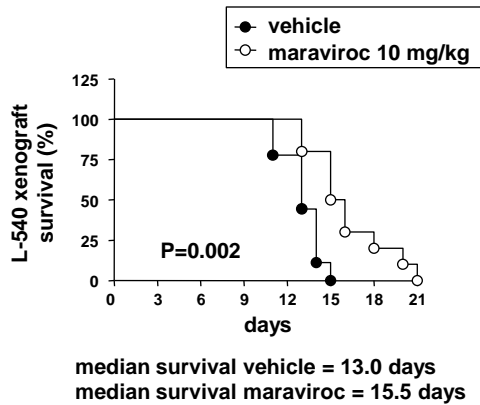
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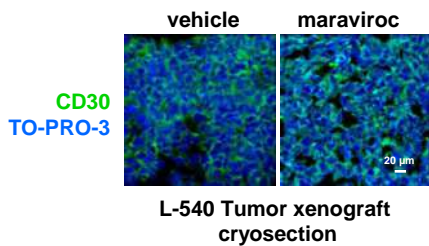
S7D



S7B



S7C



Supplementary Figure S7. Maraviroc slows tumor xenograft growth, enhances mice survival and reduces TAM infiltration. (A) Representative photographs of tumors excised from mice bearing L-540 xenografts treated or not with 10 mg/kg maraviroc. (B) Kaplan-Meier survival analysis of mouse xenograft models of L-540 cHL cells treated with 10 mg/kg maraviroc. Survival was estimated using a predefined cutoff of 800 mm³ as a surrogate (log-rank test). (C) Immunofluorescence staining of cHL cells (CD30+) in L-540 tumor xenograft cryosections from vehicle-treated and maraviroc-treated mice. Nuclei were stained with TO-PRO-3 dye. Images were acquired using a confocal microscope (Leica DM IRE2). (D) Immunofluorescence staining of intratumoral TAMs (CD68+ cells) in tumor cryosections from vehicle-treated and maraviroc-treated L-540 and L-428 xenograft mice. Nuclei were stained with TO-PRO-3 dye.